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(54) Title: MULTIPLE MATRICES FOR ENGINEERED TISSUES

(57) Abstract

A method of producing a blood vessel prosthesis, using multiple matrices containing a freeze–dried, acid–precipitated co–polymer of collagen, and a glycosaminoglycan, such that the matrix exhibits less quaternary structure than native collagen and therefore the matrix, *in vivo*, activates platelets to a lesser extent than native collagen.

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MULTIPLE MATRICES FOR ENGINEERED TISSUES

BACKGROUND OF THE INVENTION

Description of Prior Art

Small diameter vascular grafts often fail for one of two reasons: thrombogenicity or neointimal hyperplasia. Thrombogenicity of the graft leads to formation of a blood clot, acute closure of the graft and, thus, tissue ischemia. Neointimal hyperplasia, also known as intimal hyperplasia, is the proliferation of the smooth muscle cells (SMCs) and excessive production by these cells of extracellular matrix. Generally, this phenomenon is seen at the sites of anastomoses, that is, where the vascular graft is sutured to the parent or recipient vessel. It is also the phenomenon described by restenosis, that is, closure of a vessel after some intervention (e.g., PTCA).

In both restenosis and anastomotic hyperplasia, the SMCs adopt a synthetic phenotype; they proliferate, migrate, and secrete more matrix than is appropriate. Some of this phenotypic shift is due to a shift in signal transduction by surface integrins on the SMCs, especially those specific for the adhesion proteins, laminin and fibronectin.

Summary of the Invention

One aspect of the invention features a method of producing a multilayered cellular structure, including (i) forming a plurality of matrices; seeding at least one of the matrices with living cells; and (iii) assembling the matrices into a single multilayered cellular structure. In one preferred embodiment, the matrices are tubular and of different diameters. In related embodiments, the matrices include a copolymer of collagen and a glycosaminoglycan, and the living cells include smooth muscle cells, endothelial cells, fibroblasts, or any combination thereof. In another related embodiment, the matrices differ in their mechanical properties. In various preferred

embodiments, the mechanical property which differs between matrices is pore size, pore orientation, mean molecular weight between crosslinks (Mc), or percent porosity (void fraction). In yet another related embodiment, the matrices contain different amounts of elastin, laminin, fibronectin, or Tissue Factor Pathway Inhibitor (TFPI), or any combination thereof.

In a second aspect, the invention features a method of producing a prosthetic blood vessel, including the steps of (i) forming a plurality of tubular matrices of different diameters; (ii) seeding at least one of the tubular matrices with living cells; and (iii) assembling the tubular matrices into a single, concentric multilayered tube.

In a third aspect, the invention features a method of producing a prosthetic blood vessel, including the steps of (i) forming a plurality of matrices; (ii) seeding at least one of the matrices with living cells; (iii) shaping the matrices to form tubular matrices of different diameters; and (iv) assembling the tubular matrices into a single multilayered tube.

In a fourth aspect, the invention features a method of producing a prosthetic blood vessel, including the steps of (i) forming a tubular matrix; and (ii) seeding the tubular matrix with living cells. In a preferred embodiment, the matrix includes a co-polymer of collagen and a glycosaminoglycan. In a related embodiment, the matrix also includes elastin, laminin, fibronectin, or Tissue Factor Pathway Inhibitor (TFPI), or any combination thereof In another preferred embodiment, the living cells include smooth muscle cells, endothelial cells, or any combination thereof.

In a fifth aspect, the invention features a method of producing a prosthetic blood vessel, including the steps of (i) forming a matrix; (ii) seeding the matrix with living cells; and (iii) shaping the matrix to form a tube. In a preferred embodiment, the matrix includes a co-polymer of collagen and a glycosaminoglycan. In a related embodiment, the matrix can also include elastin, laminin, fibronectin, or Tissue Factor Pathway Inhibitor (TFPI), or any

combination thereof. In another preferred embodiment, the living cells include smooth muscle cells, endothelial cells, or any combination thereof.

In a sixth aspect, the invention features a matrix including a co-polymer of collagen and glycosaminoglycan, and also includes at least two of the following: elastin, laminin, fibronectin, and Tissue Factor Pathway Inhibitor (TFPI). In another preferred embodiment, the glycosaminoglycan is chondroitin sulfate or heparin. In yet another preferred embodiment, the collagen is type I or type IV collagen. In another preferred embodiment, the co-polymer of collagen and glycosaminoglycan is acid precipitated. In other preferred embodiments, the matrix also includes TFPI, elastin, laminin, and/or fibronectin.

In a seventh aspect, the invention features a multilayered blood vessel prosthesis which includes (i) an inner layer comprising type I collagen, type IV collagen, GAG, elastin, and laminin; and (ii) an outer layer comprising type I collagen, GAG, elastin, and fibronectin. In preferred embodiments, the inner layer is seeded externally with smooth muscle cells and is seeded internally with endothelial cells. In other preferred embodiments, the outer layer is seeded internally with smooth muscle cells and is seeded externally with fibroblasts. In yet another preferred embodiment, the multilayered blood vessel prostheses also includes at least one medial layer comprising collagen, GAG, elastin, and fibronectin. In a related embodiment, the medial layers are seeded internally and externally with smooth muscle cells.

"Biocompatible polymers" include poly-N-acetylglucosamine (chitin), polyurethanes, polyether block amides, fluoropolymers such as polytetrafluoroethylene, polyethylene, polyester, and polyethylene terephthalate. Biocompatible polymers also include collagen-based polymers, with or without associated glycosaminoglycans, elastin, fibronectin, laminin, or combinations thereof. Any of the 20-odd collagens thus far identified can be used; those most preferred are Type I and Type IV collagens. A biocompatible polymer is intended to contact cells, biological fluids, and preparations derived from cells.

Cells include individual cells; animal tissues such as blood, muscle, nerves, tendons, cartilage, bone, and vasculature including veins, arteries, valves, placental and umbilical material, and organs of animals; and tissues of plants. A biocompatible polymer of the invention is generally nontoxic. In some cases, the polymer can be designed to release, leech, degrade, or selectively bind biologically active substances, and direct favorable cellular processes.

A "bioresorbable material," in amounts used with the invention, can be degraded in, or by, the body without producing unacceptable levels of harmful metabolites or stimulation of adverse immune responses including cellular proliferation, calcification, or synthesis of excessive fibrotic tissue. Preferably, the degradation occurs gradually, at a predetermined rate, to allow fibroblasts, endothelial cells, or other vascular, muscular, or other tissue to replace the bioresorbable material. The time interval can be days, weeks, months or years. Moreover, the rate of degradation can be regulated by regulating the matrix components.

"Compliance" is a measure of the degree to which a material stretches as a result of an applied stress. For closed curved structures in general, compliance is defined as a change in volume of the structure divided by the change in pressure necessary to cause the change in volume. As defined by Abbot (Biological and Synthetic Vascular Prostheses, J.C. Stanley (ed), Grune and Stratton, N.Y., 1982), compliance is the percent change in diameter per unit of pressure. The compliance of the prosthesis should closely match that of the adjacent living tissue to which the prosthesis is being attached. For small diameter vascular grafts, compliance is generally between 4% and 12%, and preferably between 6% and 9%. 4% compliance means that an artery or vascular graft stretches 4% as a result of the difference between systolic and diastolic pressures--that is, at 120mm Hg, a graft or artery would have a diameter that is 4% larger than at 80mm Hg. For convenience, one may adopt

the value of the difference between systolic and diastolic pressures as the unit of pressure.

The collagen material, as precipitated with a glycosaminoglycan, is nearly insoluble in water (or saline) at physiologic pH prior to crosslinking. The crosslinking makes it less susceptible to degradation. Crosslinking reduces aqueous solubility of a collagen-bound material and increases fracture stress and resistance to enzymatic degradation (for example, by collagenase, which cleave the collagen into smaller, soluble fragments). Thus, increasing crosslinks make the graft persist longer *in situ*. Crosslinking conditions can be selected to provide the desired level of crosslink density, to reduce the surface functional groups, or otherwise modulate mechanical or biological properties. Glycosaminoglycans ("GAG") are a class of sulfated sugars that often are components of glycoproteins, and that become such when precipitated with collagen as described above. Examples of GAGs include, but are not limited to, chondroitin-6-sulfate, chondroitin-4-sulfate, heparin sulfate, dermatan sulfate, keratin sulfate, chitosan, hyaluronic acid, and heparin. Combinations of glycosaminoglycans can also be used.

"Porosity" is the estimate or index of the ratio of the void within a material to the total volume occupied by the material including the voids, expressed as a percentage void to the total volume.

The invention provides improved vascular grafts which resist failure, particularly at anastomoses. The invention also provides vascular grafts with excellent mechanical and biological properties in a much shorter time period than is possible by other methods.

Other features and advantages of the invention will be apparent from the following description and from the claims.

Detailed Description

The invention features tubular or bifurcated matrix analogs constructed of a biocompatible polymer matrix analog which can be comprised of type I and

type IV collagen, a glycosaminoglycan such as chondroitin sulfate or heparin sulfate, elastin, an adhesion protein such as laminin, or fibronectin, and Tissue Factor Pathway Inhibitor (TFPI).

In a particularly preferred embodiment, the matrix includes collagen/GAG, precipitated in an acidic medium (pH~4.25) and freeze dried. This process yields a collagen copolymer largely lacking the banding pattern (64nm) seen using Scanning Electron Microscopy (SEM) that is indicative of quaternary structure in the native collagen macromolecule. This quaternary structure is associated with platelet activation. Thus, a collagen matrix of the invention lacking this quaternary structure will not activate platelets to a significant extent. Further, the inclusion of Tissue Factor Pathway Inhibitor (TFPI), an endogenous protease inhibitor, blocks the other enzymatic cascade which leads to thrombus formation. TFPI can also be absorbed onto the matrix after the other components are precipitated and cross-linked. Thus, by combining an acidic precipitate of collagen/GAG with TFPI, both pathways of thrombus formation are inhibited and the resulting conduit is largely non-thrombogenic. In some instances, it is desirable not to include, or include at a reduced concentration, the TFPI in the outermost portion of the vascular graft. It is also desirable in some cases to include adhesion proteins such as laminin or fibronectin, to regulate SMC phenotype. Elastin can also be included to increase compliance and to increase fracture toughness. Finally, it is in some cases desirable to retain some of the quaternary structure of collagen in the outermost portions of the graft as well. These modifications of the outer portion of the graft are intended to allow or promote normal healing, particularly at the suture tracts, while leaving the inner surface of the matrix essentially non-thrombogenic.

The extracellular matrix (ECM) analog can be formed into at least two tubes, sized such that one tube fits snugly inside the other. The tubes can be manufactured such that the chemical or physical compositions, or both, are

different in each of the tubes. It is particularly useful to construct the inner tube such that the pores in the ECM are smaller than those in the outer tube. For example, it can be desirable for the inner tube to have pores of between one and 10 microns in average size, while the outer or subsequent tube can be optimal with pores that average 40-80 microns in diameter. Additionally, the inner tube can be much thinner than the outer tube, and the inner tube can be also have a higher concentration of elastin.

Collected endothelial or SMCs, after isolation and sufficient growth, a then seeded onto one or more of the tubes. Some of the tubes can be seeded with SMCs internally and externally, while others are only seeded externally. Additionally, the inner-most tube can be seeded externally with SMCs, then cultured for a few days, then seeded internally with endothelial cells. The tubes are eventually assembled and further grown, yielding a construct of concentric layers of tissue containing endothelial cells and SMCs. Fibroblasts can be added to the outermost surface for the final culture period, or it can be beneficial to construct a similar adventitial layer comprised of ECM and fibroblasts, and then assemble the adventitial layer onto the medial and intimal layers. This method yields an arterial graft with excellent mechanical and biological properties in a much shorter time period than is possible by other methods.

In an alternate procedure, the matrices are formed into sheets. The sheets can be manufactured such that the chemical or physical composition, or both, are different for each sheet. Onto these sheets, endothelial or SMCs are seeded. After culturing, the matrix sheets are shaped into tubes. The matrix sheet can be overlaid and allowed to attach to each other prior to forming tubes. Alternatively, the sheets can be shaped individually into tubes of different sizes and then assembled into a multicellular structure.

When culturing the cells on the matrix tube(s), after the cells have become fully attached to the matrices, culture medium can be forced through

the lumen of the tube in a pulsatile fashion in addition to bathing the tube in the culture medium. This will orient the SMCs in a circumferential direction, as in a natural artery. The pulsatile flow will also cause the endothelial cells to adopt an elongate, 'cobblestone' appearance, as in a normal artery. The cells on the matrices can be made to produce more matrix proteins than usual by culturing the cells in the presence of ascorbic acid. This yields a high-strength prosthesis faster than would be otherwise possible. This also leads to more rapid integration of the concentric layers once they are assembled. After the layers are sufficiently fused, the arterial analog is then packaged for sterile transfer to the operation room, where it is implanted in the patient.

The Matrix Composition

The matrix can include (i) a glycosaminoglycan, (ii) a collagen, (iii) an integrin receptor ligand such as laminin and fibronectin, (iv) elastin, and (v) TFPI. Each of these components is described further.

A matrix can include between 1% and 10% glycosaminoglycan (GAG) by dry weight, and preferably between 3% and 8% GAG by dry weight, such as 4% or 7%. A preferred GAG is chondroitin-6-sulfate.

A matrix can include between 50% and 99% collagen, such as Type IV or Type I collagen. The degree of crosslinking of the collagen determines the mechanical and bioresorptive properties of the matrix. Increases in the collagen/elastin ratio will decrease elasticity and, to a minor extent, decrease the resorption rate. The resorption rate primarily is determined by the mean molecular weight between crosslinks. The limit for this value is a function of the GAG content. How close one comes to that limiting value is determined by the processing after precipitation. Thus, two grafts with very different compositions could have identical crosslink densities (Mc), which would yield similar resorption rates.

A matrix can include between about 0.0001% and 0.1 % by dry weight of the integrin receptor ligand, and preferably between 0.001% and 0.01%.

Preferred integrin receptor ligands are laminin and fibronectin. Laminin and fibronectin are both adhesion proteins which link the matrix with SMCs via the surface integrins on those cells. Where laminin is the integrin receptor ligand present on the matrix, the laminin causes SMCs to migrate and undergo the phenotypic shift in from the desired contractile type to the undesirable synthetic type. For some grafts, or portions of grafts, the opposite result is desired. In these instances, the matrix ligand is fibronectin, which causes the SMCs to remain or become contractile, and, thus, to not migrate and proliferate. In sum, the composition of the matrices of the invention can be varied to control SMC phenotype.

TFPI can be included in the matrix in amounts to at least partially inhibit thrombus formation. TFPI in amounts up to 0.5% of the matrix weight can be used, preferably between 0.00 1% and 0. 1%.

A matrix can also include elastin. Elastin is added to the matrix to increase its compliance and to increase fracture toughness. Up to 30% by weight elastin can be used, preferably between 5 and 20%.

The components of the matrix and the method of crosslinking will determine pore size. Pore size can be used alone or in combination with ligands to assist in directed migration of cells toward a desired region of the shaped mate. The shaped material can be, for example, a sheet, a tube, a bifurcated tube, or tapered tube. In one example, one portion, such as an inner surface of a tube, has smaller pores than another surface, such as an outer surface of a tube, or *vice versa*. For example, if the shaped material is a vascular graft, it is desirable to encourage migration of SMCs toward the outer surface or middle portion of the graft, and migration of vascular endothelial cells along the inner surface of the graft.

Matrices can also include other substances on the surface of the shaped polymer composition, or throughout the polymer composition. Examples include ligands which promote selective migration of desired cells towards or

adherence to a surface, or which reduce migration or adherence of undesirable cells, such as platelets. For example, platelet activation is inhibited by the use of prostacyclin, a prostacyclin analog, or other platelet-activating factor antagonists. Other targeting, repelling, or nutrient substances include VEGF, TGF-beta, or any other desired growth factor or inhibitor.

The Cells

The three main cell types that make up an artery are SMCs, endothelial cells, and fibroblasts. There are many possible sources for these cells. For example, the cells can be collected from the patient and cultured. Alternatively, the cells can be allogenic, coming from donated placental or umbilical tissue. The cells can arise from embryonic stem cells which have become SMCs, endothelial cells, or fibroblasts (or precursors to these cells) through a cell-intrinsic program, response to exogenously-applied factors, or a combination of both. The cells can be increased in number through extended culturing prior to being seeded onto the matrix.

Spatial Regulation of Cell Types

SMCs, endothelial cells, and fibroblasts respond differently to matrix and adhesion proteins. Moreover, different regions of a graft require different proportions of cell types. Thus, the amount of fibronectin and/or laminin in any region of the graft can be varied according to the needs of that region. Thus, the concentrations of these proteins will vary depending on the properties required at individual radial and longitudinal positions in the graft. Thus, it is desired that endothelial cells eventually line the inner surface of the graft, and therefore collagen/GAG/laminin is used to mimic basement membrane in this region. Small pores at the inner surface of the graft will prevent the SMCs from contacting the laminin, and thus discourage SMC migration and proliferation.

In the medial portion of the graft, some proliferation and migration can be desired, as it is beneficial to have the graft rapidly healed. To accomplish this, the medial matrix can have both fibronectin and laminin, and the relative

amounts of the two adhesion proteins can vary with the length of the graft. For example, because of the problem with anastomotic hyperplasia, it is desirable to have no laminin or fibronectin at the ends of the graft. Moving towards the central portion of the graft, it can be beneficial to have higher laminin and fibronectin content in order to encourage the SMCs to continue to migrate, so that they can eventually populate the entire graft.

Finally, the outer portion of an artery, the adventitia, is largely populated by fibroblasts. To encourage their growth and infiltration, a third composition of the ECM analog can be employed in this outer region of the graft. Though not as responsive as the medial smooth muscle layer, this outer fibroblast layer is critical in that the collagenous matrix of the adventitia provides much of the ultimate mechanical strength of the artery. Thus, a thin layer of relatively dense collagen with fibronectin, perhaps with little GAG, can be appropriate for the outer section of the graft. This outer layer must serve two functions initially; it must provide the ultimate strength to prevent rapture of the graft while other cells are migrating, and it must also provide a hospitable environment for proliferation and matrix turnover by fibroblasts.

In summary, the inner portion of the graft will have high laminin content and small pores. The small pore size prevents the SMCs from interacting with the laminin, thus encouraging endothelialization, and discouraging migration, proliferation and synthesis by the SMCs. The medial portion of the graft will have larger pores and a mixture of laminin and fibronectin, which will lead to control of the phenotype of the SMCs and also of the eventual population of the entire medial portion of the graft with SMCs of the contractile phenotype. The outer portion of the graft will likely be more dense, and will have more fibronectin than the other two regions if it is needed to encourage fibroblast growth.

<u>Vascular grafts</u>

The invention features prosthetic devices and methods for producing such devices for cardiovascular grafting. Shapes for prosthetic devices in the cardiovascular system include valves, elbows, T-joints, tubes, branched tubes, and tubes with varying or tapering diameters. Cardiovascular prostheses include small and large diameter vascular prostheses, aortic valves, venous valves, mitral valves, and prostheses used in techniques such as coronary artery bypass grafting, coronary stenting, coronary stent-grafting, transvascular shunting, transmyocardial revascularization, and endovascular grafting of aortic, peripheral, or carotid disease.

Cardiovascular prostheses preferably approximate the mechanical and performance properties of the natural tissue, particularly puncture-resistance, kink resistance, self-sealing, non-thrombogenicity, and infection resistance. Additional physical characteristics include suture retention, water permeability, integral water leakage, water entry pressure, circumferential tensile strength, longitudinal tensile strength, burst strength, diaphragm burst strength, probe burst strength, pressurized burst strength, strength after repeated puncture, usable length, relaxed internal diameter, pressurized internal diameter, and wall thickness. International quality control standards and safety testing procedures are known in the art and are described, for example, in ISO/DIS 7198 (ISO/TC 1501SC 2 N169), CEN/TC 28/WG 3/TF2, and the Revised A.A.M.I. Standard.

In one example, a small diameter vascular graft has an inner diameter between 1.0 mm and 6.0 mm, preferably between 1.5 and 4.0 mm; and a wall thickness between 0.25 mm and 3 mm, preferably between 0.5 mm. and 1.5 mm.

A vascular graft can have branches (e.g., a bifurcated or trifurcated graft), or tapering ends such that the diameter at one end is smaller than the diameter at another end, or have both branches and tapering portions. The vascular conduit can have a variable modulus along the length, without varying the diameter. One example of a bifurcated graft is between 3 and 9 inches long,

preferably between 5 and 8 inches long, and has a proximal diameter of about 6 mm at the end to be attached to the descending aorta. The two distal ends have diameters of about 2.5 mm and are to be attached to the coronary arteries. Preferably, the proximal end will stretch more than the distal ends, as the elasticity of each end should match the natural tissue (aortic and coronary arterial, respectively). Usually the proximal end of a graft is sewn to the ascending aorta.

Examples of grafts, produced from the methods described herein, are described next.

Example I

A matrix tube is formed by creating an acidic dispersion of type I collagen in acetic acid at pH of ~4.2. Chondroitin-6-sulfate, also in acetic acid, is slowly added to the collagen suspension with mixing. The precipitate is then centrifuged to concentrate, and the concentrated slurry is injected into a mold, which defines an annular space of 2.5 mm. ID and 3.0 mm OD. The mold is submerged in a bath of liquid nitrogen, and rapidly frozen. The mold is then vented, and placed in a freeze-drying chamber. The aqueous solution of acetic acid is sublimed, and the collagen/GAG matrix is stabilized by the formation of crosslinks by a dehydration reaction. After sublimation is complete, further crosslinking is achieved by placing the matrix in a vacuum oven at 105°C for several hours, preferably 24 hours. The matrix is then crosslinked further by immersing it in a 0.5% glutaraldehyde bath at 37°C for 4 hours. The matrix is then rinsed with sterile water until all residue of unreacted glutaraldehyde is removed.

The matrix is then seeded with SMCs obtained from a donated human umbilical artery by sealing one end of the tube and injecting a suspension of cells into the lumen of the tube, thus forcing cells into the matrix. Cells are also deposited externally onto the tube. The tube is then cultured in DMEM with 10% fetal bovine serum and 50 μg/ml sodium ascorbate in a CO_2 incubator,

92% air, 8% CO_2 at 37° C. After 24 hours, the tube is connected to a pulsatile apparatus, which forces the same culture medium through the lumen of the tube with slowly increasing force. After one week, this tube is placed within a series of slightly larger, similarly constructed tubes, and this assembly is then returned to the incubator and connected to the pulsatile apparatus for further culturing of two weeks. A similar layer of matrix seeded with fibroblasts is added externally, and after one more week of culture, the graft is ready for implantation. Note that endothelial seeding is not required because the quaternary structure of collagen has been abolished and the graft is thus non-thrombogenic. Note also that allogenic SMCs are employed in the absence of allogenic endothelial cells. SMCs are not able to activate T-cells, and so in the absence of endothelial cells, which present high levels of HLA molecules, the allogenic SMCs do not elicit a strong immunological response. The assembled tube is now placed in chilled hypothermosol® (Mediatech), and delivered to the hospital, where it is implanted in the patient.

Example 2

A flat sheet matrix is made by pouring the slurry onto the steel sheet, and spreading the slurry to a uniform thickness of 0.005 inches. This sheet is then placed on the shelf in the freeze dryer, and rapidly frozen. Subsequent steps are as in Example 1, until SMCs are seeded onto the sheet at a density of 3-5 x 10^4 cells/cm^2. After one week of culture under the conditions described in Example 1, the sheet is cut into strips one inch wide, and these strips are then helically wound around a Teflon® mandril of diameter 2.5 mm. After twenty more days in culture, the mandril is removed, and the assembly is connected to a pulsatile apparatus for another week of culture, after which time a sheet that had been seeded with fibroblasts is wrapped around the outside of the graft assembly. Further culture for two weeks yields a graft competent for implantation.

What is claimed is:

1. A method of producing a multilayered cellular structure, said method comprising

(a) forming a plurality of matrices;

(b) seeding at least one of said matrices with living cells; and

(c) assembling said matrices into a single multilayered cellular structure.

2. The method of claim 1, wherein said matrices are tubular and of different diameters and wherein said multilayered cellular structure is tubular.

3. The method of claim 1, wherein said matrices comprise a copolymer of collagen and a glycosaminoglycan.

4. The method of claim 1, wherein said living cells comprise muscle cells, endothelial cells, fibroblasts, or any combination thereof.

5. The method of claim 1, wherein said matrices differ in their mechanical properties.

6. The method of claim 1, wherein said matrices contain different amounts of elastin, laminin, fibronectin, or Tissue Factor Pathway Inhibitor (TFPI), or any combination thereof.

7. A method of producing a prosthetic blood vessel, said method comprising:

(a) forming a plurality of tubular matrices of different diameters;

(b) seeding at least one of said tubular matrices with living cells;

(c) assembling said tubular matrices into a single, concentric multilayered tube, said tube being a prosthetic blood vessel.

8. A method of producing a prosthetic blood vessel, said method comprising

(a) forming a plurality of matrices;

(b) seeding at least one of said matrices with living cells;

(c) shaping said matrices to form tubular matrices of different diameters; and

(d) assembling said tubular matrices into a single multilayered tube being a prosthetic blood vessel.

9. A method of producing a prosthetic blood vessel, said method comprising:

(a) forming a tubular matrix; and

(b) seeding said tubular matrix with living cells, said tubular matrix seeded with living cells being a prosthetic blood vessel.

10. A method of producing a prosthetic blood vessel, said method comprising:

(a) forming a matrix;

(b) seeding said matrix with living cells; and

(c) shaping said matrix seeded with said living cells to form a tube being a prosthetic blood vessel.

11. The method of claim 9 or 10, wherein said matrix comprises a polymer of collagen and a glycosaminoglycan.

12. The method of claim 11, wherein said matrix further comprises elastin, laminin, fibronectin, or Tissue Factor Pathway Inhibitor (TFPI), or any combination thereof.

13. The method of claim 9 or 10, wherein said living cells are smooth muscle cells, endothelial cells, or any combination thereof.

14. A matrix comprising a co-polymer of collagen and glycosaminoglycan, wherein said matrix further comprises laminin, fibronectin, or Tissue Factor Pathway Inhibitor (TFPI), or any combination thereof.

15. The matrix of claim 14, wherein said glycosaminoglycan is chondroitin sulfate or heparin.

16. The matrix of claim 14, wherein said collagen is type I or type IV collagen.

17. The matrix of claim 14, wherein said co-polymer of collagen and a glycosaminoglycan is acid precipitated.

18. The matrix of claim 14, wherein said matrix comprises Tissue Factor Pathway Inhibitor (TFPI) in an amount sufficient to decrease thrombus formation *in vivo*.

19. The matrix of claim 14, wherein said matrix further comprises elastin.

20. The matrix of claim 14, wherein said matrix comprises at least one of the adhesion proteins laminin and fibronectin in an amount sufficient to affect the phenotype of smooth muscle cells.

21. A multilayered blood vessel prosthesis comprising

(a) an inner layer comprising type I collagen, type IV collagen, C elastin, and laminin; and

(b) an outer layer comprising type I collagen, GAG, elastin, and fibronectin.

22. The multilayered blood vessel prosthesis of claim 21, wherein said inner layer is seeded externally with smooth muscle cells.

23. The multilayered blood vessel prosthesis of claim 21, wherein said inner layer is seeded internally with endothelial cells.

24. The multilayered blood vessel prosthesis of claim 21, wherein said outer layer is seeded internally with smooth muscle cells.

25. The multilayered blood vessel prosthesis of claim 21, wherein said outer layer is seeded externally with fibroblasts.

26. The multilayered blood vessel prosthesis of claim 21, further comprising at least one medial layer comprising collagen, GAG, elastin, and fibronectin.

27. The multilayered blood vessel prosthesis of claim 26, wherein said medial layers are seeded internally-and externally with smooth muscle cells.

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(54) Title: MULTIPLE MATRICES FOR ENGINEERED TISSUES

(57) Abstract: A method of producing a blood vessel prosthesis, using multiple matrices containing a freeze-dried, acid-precipitated co-polymer of collagen, and a glycosaminoglycan, such that the matrix exhibits less quaternary structure than native collagen and therefore the matrix, *in vivo*, activates platelets to a lesser extent than native collagen.

INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER

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US CL : 623/1.41, 1.44

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 623/1.41, 1.44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X, P	US 5,879,383 A (BRUCHMAN et al.) 09 March 1999, col. 5, lines 24-26 and 55-64; col. 6, lines 45-53; col. 9, lines 62-67; col. 10, lines 1-11 and 47-51; col. 13, lines 39-55; col. 15, lines 60-67; col. 16, lines 45-55; col. 17, lines 21-31 and 53-67; and Figs. 1-9.	1-20
A	US 3,938,524 A (SPARKS, deceased et al.) 17 February 1976, col. 2, lines 51-61.	21-27
A	US 5,336,256 A (URRY) 09 August 1994, col. 3, lines 4-41.	21-27
A	US 5,716,394 A (BRUCHMAN et al.) 10 February 1998, col. 5, lines 59-67.	21-27

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5,735,897 A (BUIRGE) 07 April 1998, col. 2, lines 49-67.	21-27

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